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Organizing Activity of Wingless Protein in Drosophila

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Summary

The adult appendages of Drosophila are formed from imaginal discs, sheets of epithelial cells that proliferate during larval development and differentiate during metamorphosis. wingless (wg, DWnt-1) protein, a putative signaling molecule, is expressed only in prospective ventral cells in each of the leg discs. To test the role of *wg*, we have generated randomly positioned clones of cells that express wg protein constitutively. Clones that arise in the prospective ventral portions of the leg discs develop normally. In contrast, dorsally situated clones give rise to ventrolateral patterns and exert a ventralizing influence on neighboring wild-type tissue. We propose that wg protein organizes leg pattern along the dorsoventral axis by conferring ventral positional information within the disc.

Introduction

The process of pattern formation depends on the ability of single cells or cell groups to respond differently according to their position within a primordium. Yet in most cases, we know little about the molecules or mechanisms responsible for generating the spatial information to which these cells respond. One exception is the generation of body pattern in early Drosophila embryos. In this case, the primary determinants have been identified, and their molecular natures and modes of action are at least partly understood (e.g., reviewed by St Johnston and Nüsslein-Volhard, 1992). However, early Drosophila embryos are syncytial; hence, spatial signals arise and diffuse within a single cell or in the fluid layer that surrounds it. This differs from most other patterning systems, in which spatial signals arise in populations of cells composing organs or appendages. By comparison, our understanding of pattern formation in these other systems remains poor.

One way spatial information could be generated within a tissue is that a small group of cells might express a factor that could influence the behavior of neighboring cells. Such a factor could accumulate in the form of a gradient and act as a morphogen capable of triggering distinct responses as a function of its changing concentration (e.g., Dalcq, 1938; Lawrence, 1972; Sander, 1975; Tickle et al., 1975). Alternatively, it might stimulate neighboring cells in an all-or-none fashion to express a second factor, which in turn stimulates the expression of additional factors in other cells, thereby generating a chain of inductive signals (e.g., Spemann, 1938; Rose, 1952; Jacobson and Sater, 1988; Tomlinson, 1988).

Candidates for spatial signals have been suggested in a number of systems, e.g., retinoic acid in vertebrate limbs (Tickle et al., 1982; Thaller and Eichele, 1987; reviewed by Eichele, 1989), small peptides in hydra (Schaller et al., 1979; Schaller and Bodenmüller, 1981), and activin and noggin in Xenopus embryos (Green and Smith, 1990; Smith and Harland, 1992). However, in each case, uncertainties remain about whether the factor is expressed in the appropriate cells at the relevant time and whether its activity is normally responsible for organizing the pattern of surrounding cells.

Another candidate for a diffusible factor that governs spatial pattern is the protein product of the Drosophila segment polarity gene *wingless* (*wg*; Sharma and Chopra, 1976; Nüsslein-Volhard and Wieschaus, 1980; also called *DWnt-1*, Nusse and Varmus, 1992). Several findings suggest that *wg* functions as an intercellular signal involved in pattern formation. First, mutations that reduce or eliminate *wg* function have global effects on cell pattern within embryonic segments and in the adult appendages, even though wg protein expression is restricted to a subset of cells within each primordium (Nüsslein-Volhard and Wieschaus, 1980; Baker, 1987, 1988; Martinez Arias et al., 1988; van den Heuvel et al., 1989; Peifer et al., 1991). Second, *wg* activity in some embryonic cells is required for maintaining the expression of the *engrailed* (*en*) gene in adjacent, nonexpressing cells (Dinardo et al., 1988; Martinez Arias et al., 1988; Heemskerk et al., 1991; Bejsovec and Martinez Arias, 1991). Third, both wg protein and its vertebrate homolog, Wnt-1, appear to be secreted in tissue culture and in vivo (Papkoff et al., 1987; van den Heuvel et al., 1989; Papkoff and Schryver, 1990; Bradley and Brown, 1990; González et al., 1991; Chakrabarti et al., 1992; reviewed by Nusse and Varmus, 1992). These findings raise the possibility that wg protein expressed by defined subsets of cells may organize the pattern of surrounding cells.

To test whether the local expression of wg protein by some cells is sufficient to organize the pattern of neighboring, nonexpressing cells, we devised a general method for altering where genes are expressed in developing tissues. The gist of this technique is to use a site-specific recombinase, the yeast protein flp (Broach and Hicks, 1980; Jayaram, 1985), to fuse the promoter of one gene to the coding sequence of another. We used this approach to generate randomly positioned clones of cells that constitutively express wg protein. We show that in the imaginal leg discs, where *wg* is normally expressed only in prospective ventral cells, ectopic *wg* activity in more dorsally positioned cells reorganizes dorsoventral pattern. The expression of wg protein in a small subset of cells can therefore influence the developmental behavior of surrounding, nonexpressing cells, in a manner reminiscent of the classical embryonic organizer (Spemann and Mangold, 1924; Spemann, 1938). Further, the nature of the duplicated pat-

terns, as well as the restricted positions in which they are observed is consistent with the hypothesis that ventrally positioned cells provide a local source of wg protein that spreads dorsally and controls leg pattern.

Results

A General Method for Heritably Activating Any Gene in Any Cell

The yeast recombinase *flp* normally catalyzes recombination between homologous 700 bp target sites (termed *flp* recombination targets, or FRTs) in the 2μm minichromosome (Broach and Hicks, 1980; Jayaram, 1985). When FRTs are arranged as inverted repeats flanking a segment of DNA, each recombination event serves to reverse the orientation of the DNA lying between them. Alternatively, when the FRTs are arranged as direct repeats, *flp*-mediated recombination leads to excision of the intervening DNA and joining of the sequences on either side. Because the action of *flp* recombinase is sufficient to cause efficient site-specific recombination between FRTs in Drosophila (Golic and Lindquist, 1989), *flp*-mediated recombination between direct FRT repeats is well suited to creating gene fusions during fly development.

Figure 1 illustrates the approach we have devised to generate constitutive expression of any coding sequence in marked cells chosen at random during particular developmental stages. (A similar approach was devised independently by R. Holmgren [personal communication].) In brief, a constitutive promoter, *Act5C* (*Actin in the 5C region*; Bond and Davidson, 1986), is placed upstream of a given coding sequence (*lacZ* or *wg*) but separated from it by a segment of DNA bounded by direct FRT repeats that we call a *flp*-out cassette. If, as diagramed, the intervening DNA contains a transcriptional stop, the coding sequence will not be expressed unless the cassette is excised by *flp*-mediated recombination. Under these circumstances, a brief pulse of ubiquitous *flp* expression driven by the heat-inducible *hsp70* promoter (Lis et al., 1983) can suffice to excise the transcriptional stop, thereby activating heritable expression of the coding sequence in single cells. Because the level of ubiquitous *flp* expression depends on the severity of the heat shock applied, the frequency of excision events and hence of clones of constitutively expressing cells can be readily controlled.

To assay the effectiveness of the general approach, we first tested a hybrid gene in which the *Act5C* promoter is positioned upstream of a coding sequence for nuclear-localized β-galactosidase (*nuc-lacZ*; Riddihough and Ish-Horowicz, 1991) but separated by a *flp*-out cassette containing the *hsp70* transcriptional stop (Udvardy et al., 1985) flanked by direct repeats of a minimal FRT (Figure 1; this cassette also includes a 4.4 kb fragment containing a functional *Draf*$^+$ gene [Nishida et al., 1988] whose presence is incidental to the experiments described here). Animals carrying this gene, termed *Act5C>Draf*$^+$*>nuc-lacZ* (greater-than signs designate the flanking FRTs), as well as an *hsp70–flp* gene express *nuc-lacZ* in a heat shock–dependent fashion, with both the number and pattern of

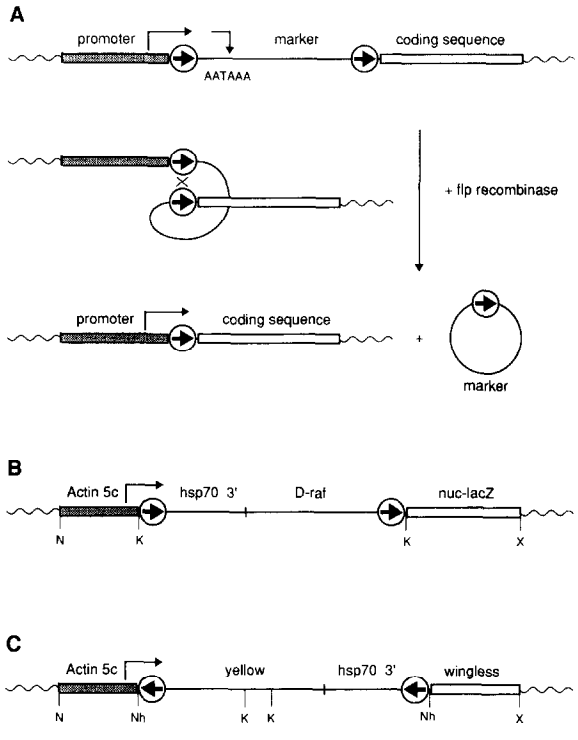

Figure 1. Heritable Activation of *lacZ* and *wg* Expression by *flp*-Mediated Recombination

(A) The *flp* recombinase mediates site-specific recombination between cis-acting FRTs. When the FRTs are arranged as direct repeats flanking a segment of DNA, recombination leads to the excision of the intervening DNA, leaving behind a single FRT. As described, our general approach is to place a constitutive promoter upstream of the first FRT, a transcriptional terminator between the FRTs, and a coding sequence downstream of the second FRT. Prior to recombination, the promoter drives expression of transcripts that terminate within the DNA bounded by the FRTs; after recombination the promoter now drives constitutive expression of the coding sequence. Note that the other product of the recombination event (closed circle) is lost upon cell division; hence, cells that inherit the fused gene can also be genetically marked if the *flp*-out cassette carries an appropriate marker gene (e.g., the y^+ gene in flies that lack activity of the endogenous gene).

(B) The *Act5C>Draf*$^+$*>nuc-lacZ* gene.

(C) The *Act5C>y*$^+$*>wg* gene. See Experimental Procedures for details. Note that the *Act5C* promoter contains two XbaI sites and each minimal FRT a single XbaI site that are not indicated. The *flp*-out cassette can be inserted in the plus or minus orientation, as shown, respectively, for the *Act5C>Draf*$^+$*>nuc-lacZ* and *Act5C>y*$^+$*>wg* genes: in the minus orientation, transcriptional termination within the *flp* gene fusion cassette depends on cryptic terminator sequences rather than on the *hsp70* terminator sequences. The minus orientation was used in the case of the *Act5C>y*$^+$*>wg* gene because we failed to obtain transformants containing the *>y*$^+$*>* *flp*-out cassette in the plus orientation.

K, KpnI; N, NotI; Nh, NheI; X, XbaI.

expressing cells depending on the timing and severity of heat shock applied. In contrast, *nuc-lacZ* expression is not observed in animals carrying only the *Act5C>Draf*$^+$*> nuc-lacZ* gene.

For example, when first instar larvae carrying both the *Act5C>Draf*$^+$*>nuc-lacZ* and *hsp70–flp* genes are subjected to a single, moderate heat shock (32°C for 30 min) and allowed to develop until late in the third larval instar,

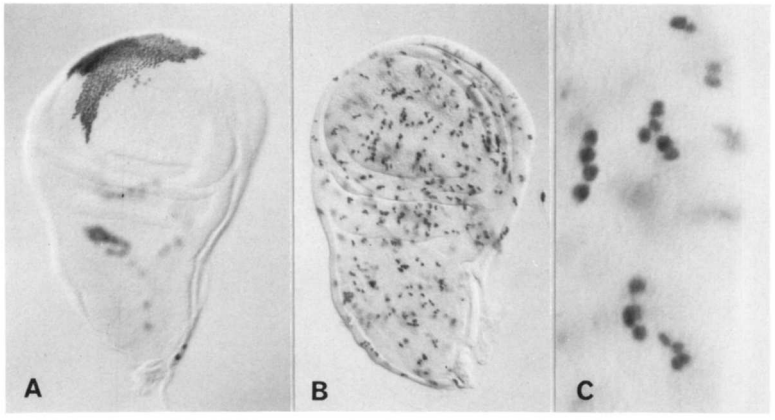

Figure 2. Clones of *Act5C>nuc-lacZ*–Expressing Cells following *flp*-Mediated Recombination

nuc-lacZ expression in wing discs obtained from late third instar *hsp70–flp/Act5C>Draf⁺>nuc-lacZ* larvae previously heat shocked for 30 min at 32°C during the first (A) or mid-third (B and C) instar. A single patch of a few hundred contiguous cells can be seen at the top of (A). Some polyploid cells belonging to the tracheae attached to this disc were also marked and can be seen, out of focus, under the middle and lower portion of the disc. In contrast, the disc shown in (B) is peppered with small patches. As shown at higher magnification in (C), the marked cells are clustered as would be expected if they are the clonal derivatives of single cells.

patches of typically a few hundred contiguous *nuc-lacZ*–expressing cells are observed in the imaginal discs (Figure 2A), whereas no such patches are found in discs of siblings not subjected to heat shock. In contrast, a heat shock of the same time and intensity administered in the middle of the third larval instar generated a high frequency of smaller patches (Figures 2B and 2C). The number of patches generated at each time also depends on the temperature and duration of the heat shock applied, higher temperature or longer heat shocks resulting in a higher patch frequency (data not shown).

Because *nuc-lacZ* expression is observed only when both the *Act5C>Draf⁺>nuc-lacZ* and *hsp70–flp* genes are present and is dependent on heat shock, we infer that it arises as a consequence of *flp*-mediated fusion of the *Act5C* promoter to the *nuc-lacZ* coding sequence. Indeed, Southern blot analysis of animals containing both hybrid genes indicates that heat shock induces the expected *flp*-mediated recombination event diagramed in Figure 1 (data not shown). Thus, we conclude that the patches of *nuc-lacZ*–expressing cells we observe following heat shock are clones of cells derived from single progenitor cells in which *flp*-mediated recombination has occurred.

wg Expression in the Leg Imaginal Discs

The imaginal discs giving rise to the legs are each formed by an in-pocketing of the embryonic epidermis to generate a bag-like monolayer of cells connected by a stalk to the larval body wall (Figure 3A). One side of this bag forms a columnar epithelium that gives rise to most of the adult cuticular structures, whereas the other side forms a squamous epithelium referred to as the peripodial membrane. Figure 3B shows the prospective fate map of the imaginal leg disc relative to the adult appendage (after Schubiger, 1971). Note that the leg telescopes out from the disc, with cells in the center forming distal structures such as the claws, while progressively more peripheral rings of cells form progressively more proximal segments of the leg. The adult leg is also subdivided into anterior and posterior compartments, each formed by separate lineage polyclones (Garcia-Bellido et al., 1973; Steiner, 1976; Lawrence and Morata, 1977). The gene *en* functions selectively in posterior compartment cells in each imaginal disc (Morata and Lawrence, 1975; Lawrence and Struhl, 1982; Busturia and Morata, 1988; Hama et al., 1990). In the leg imaginal discs, the *en* gene product is expressed in a broad domain encompassing approximately half of the leg disc, the boundary of expression running through the center of the disc, where it falls between cells destined to give rise to the anterior and posterior claws (Figure 3; see also Brower, 1986; Hama et al., 1990). In the adult leg, the anterior compartment is larger than the posterior compartment and includes both the most dorsal and most ventral portions of the appendage (Figure 3C; Lawrence et al., 1979).

Wg protein is expressed in a restricted sector of the leg disc that is fated to give rise to a ventral portion of the adult limb (Baker, 1988; Peifer et al., 1991). To clarify the relationship of this domain of expression to the adult pattern, we have examined the expression of a *wg* allele that expresses a nuclear-localized form of β-galactosidase instead of wg protein (K. Irvine and U. Gaul, personal communication) relative to that of the endogenous *en* gene. As shown in Figures 3D and 3E, *wg*-driven nuclear-localized β-galactosidase expression is observed in a ventral sector that overlaps slightly the domain of *en* expression. Comparison of this spatial relationship (Figure 3B) with the relative contributions of the anterior (A) and posterior (P) compartments to the adult leg (Figure 3C) indicates that *wg*-expressing cells normally give rise to the most ventral portion of the leg.

Clones of Cells Constitutively Expressing *wg* Can Reorganize Leg Pattern

To determine the phenotypic consequences of activating *wg* expression in cell clones positioned at random within the imaginal discs, we constructed a hybrid gene termed *Act5C>y⁺>wg*, in which the *Act5C* promoter is positioned upstream of the *wg* coding sequence but separated from it by a *flp*-out cassette that contains the *hsp70* transcriptional stop as well as the cell marker gene *yellow⁺* (*y⁺*; see Figure 1). When introduced into animals otherwise mutant for the *y* gene, the *Act5C>y⁺>wg* gene fully rescues the

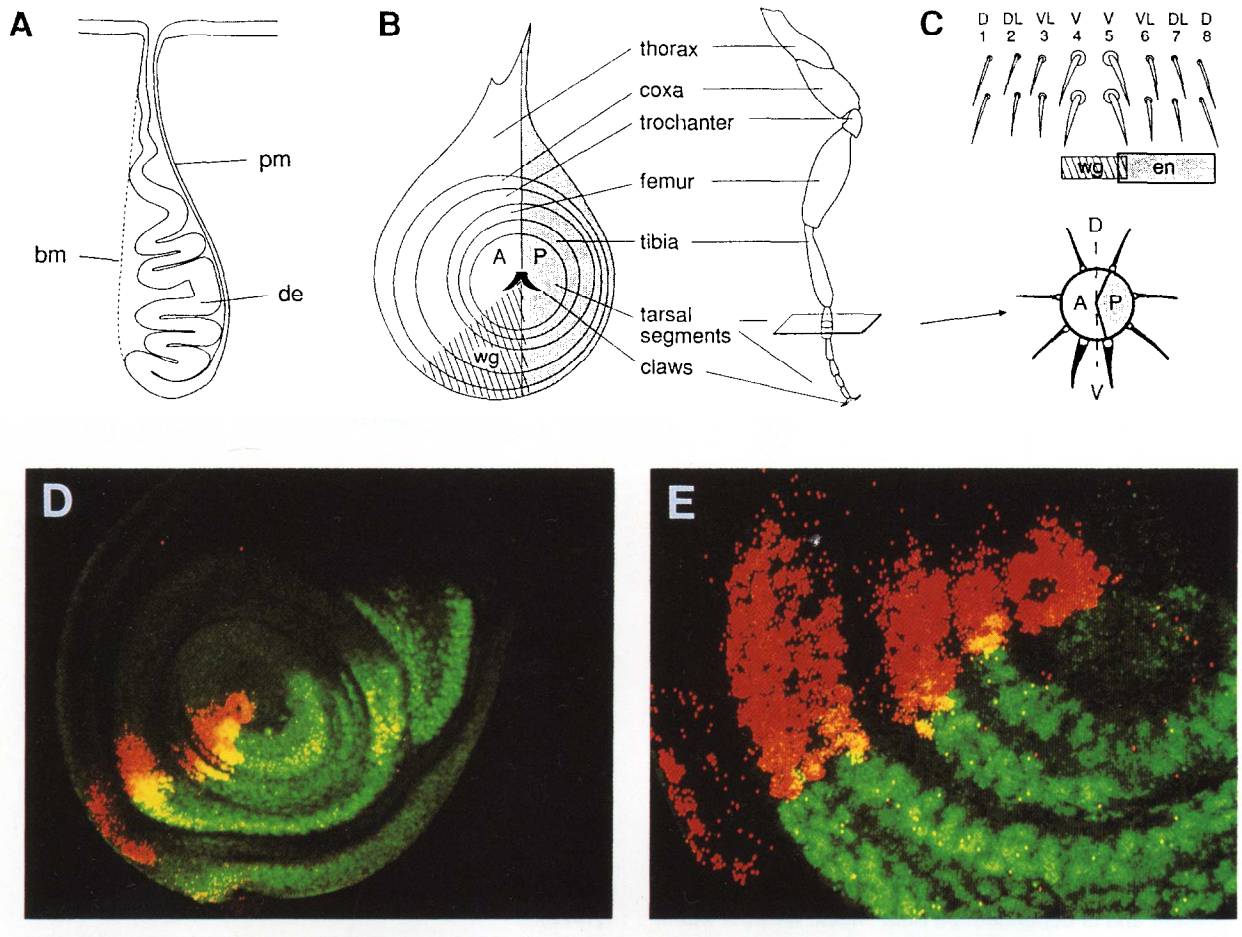

Figure 3. *wg* Expression in the Imaginal Leg Disc

(A) Diagram of a leg imaginal disc in cross section, to illustrate the relationship between the larval hypodermis (at top), disc epithelium (de), and peripodial membrane (pm), which form a continuous epithelial sheet; the extracellular basement membrane (bm) is also indicated.

(B) Diagram of the leg disc epithelium and its relation to the adult leg (from Schubiger, 1971). Note that the disc is subdivided into anterior (unstippled) and posterior (stippled) compartments, and that distal structures, such as the claws, arise from the center of the disc, while more proximal leg segments derive from more peripheral regions. The domain of *wg* expression in the ventral (lower) half of the disc is indicated by hatching.

(C) Diagram of cross section through a tarsal segment of the second leg, showing the pattern of bristles along the dorsoventral axis. The relative contributions of the anterior (A) and posterior (P) compartments are shown as unstippled and stippled, respectively (Lawrence et al., 1979). The size and shape of bristles vary along the dorsoventral axis, and the anterior compartment contributes more than the posterior compartment, including both the dorsalmost and ventralmost portions of the leg. As described in the text and diagramed above the cross section, the tarsal bristles are organized into eight columns (numbered 1–8 along the dorsoventral axis; as in Hannah-Alava, 1958; D, DL, VL, and V = dorsal, dorsolateral, ventrolateral, and ventral, respectively), of which the ventralmost two columns (4 and 5) appear to derive from *wg*-expressing cells. The relationship of *wg*-expressing cells to the adult pattern (hatched block beneath diagram) has also been confirmed by visualizing β-galactosidase expression in the legs of freshly emerged flies that express *lacZ* under the control of the endogenous *wg* promoter (data not shown).

(D and E) Low and high magnification confocal images showing wg-nuc-LacZ protein expression (red) and en protein expression (green) in imaginal leg discs. Note that the discs are oriented at an angle relative to the diagram in (B) such that the anteroposterior compartment boundary runs diagonally from the bottom left to top right corner of the field. There is a slight but significant overlap (yellow) in expression. Interpretation of this overlap is constrained by the fact that expression of the *wg-nuc-lacZ* allele has not been shown to coincide precisely with that of the endogenous wg^+ allele. Moreover, *en* expression in the mature leg disc has not been shown to coincide exactly with the compartment boundary (see Blair, 1992).

y phenotype (yellow-colored cuticular structures such as bristles and bracts) but has no other effect. However, as described below, excision of the *flp*-out cassette simultaneously joins the *Act5C* promoter to the *wg* coding sequence and eliminates the y^+ marker gene, thereby generating clones of *y Act5C>wg*-expressing cells whose descendants can be identified in the adult leg.

In our initial experiments, we subjected larvae lacking the endogenous *y* gene but carrying both the *hsp70–flp* and *Act5C>*y^+*>wg* genes to heat shocks of varying intensity at different developmental stages and then examined surviving adults for phenotypically *y* clones. A single 20 min heat shock of 32°C administered during the first or second larval instar had little effect on survival to the adult stage, and the resulting flies often contained *y* clones, some of which were associated with altered limb patterns (see below). However, flies carrying phenotypically abnormal limbs sometimes failed to emerge from the pupal case,

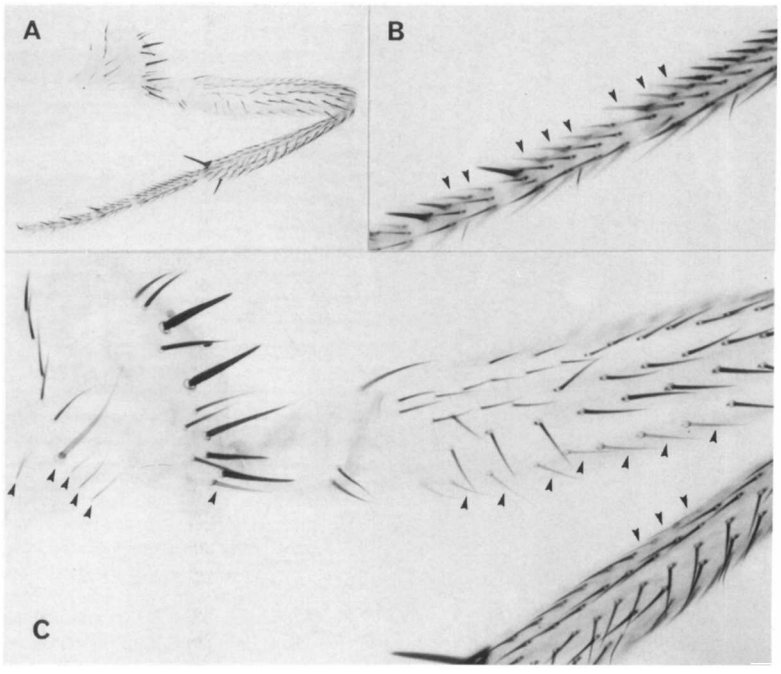

Figure 4. Phenotypically Normal *y Act5C>wg* Clone Restricted to the Ventral Portion of the Anterior Compartment of the Second Leg

(A) Overview of the leg. The clone marks a thin strip of ventral tissue extending proximodistally from the body wall through the tarsal segments.

(B) Higher magnification view of the tarsus showing that the *y Act5C>wg* clone contributes to the ventralmost column of thick, peg-like bristles (see column 4 in Figure 3C). The bristles marked with *y* and hence belonging to the clone are indicated by arrowheads.

(C) Higher magnification view of more proximal portions of the leg showing *y* bristles in ventral portions of the body wall, coxa, femur, and tibia (arrowheads). The ventral portion of the trochanter is also marked with *y* bristles, but these are out of the plane of focus.

or did emerge but became trapped in the media shortly thereafter, causing this class of progeny to be underrepresented (see Experimental Procedures). Heat shocking at higher temperatures (34°C and 35°C) during the same period caused significant pupal lethality (>50%) as well as higher frequencies of *y* clones among the surviving flies. When dissected from the pupal case, flies that had failed to emerge were usually found to have several *y* clones, some of which were associated with limb abnormalities. Few flies, if any, survived following a single heat shock of more than 35°C. The ability of heat shock to cause pupal lethality and clone induction depends on the presence of both the *hsp70–flp* and *Act5C>y⁺>wg* genes: multiple heat shocks of 37°C could be applied to larvae carrying either gene alone without significantly reducing viability or generating *y* clones. Southern blotting experiments have confirmed that *flp* activity mediates the expected recombination event between the *Act5C* promoter and the *wg* structural gene (data not shown), as in the case of the *Act5C>Draf⁺>nuc-lacZ* target gene.

Although *y Act5C>wg* clones associated with abnormal patterns were obtained in most of the adult derivatives of the imaginal discs, we have concentrated principally on clones generated in the legs. We have also limited our analysis to clones generated during early larval life (during the first and second larval instars; i.e., approximately 1–3 days after fertilization at 25°C). Clones of *y Act5C> wg* cells generated during this period typically form thin, longitudinal strips extending proximodistally through several leg segments. In this respect, they resemble *y* but otherwise wild-type clones generated by X-ray–induced mitotic recombination (e.g., Lawrence et al., 1979).

y Act5C>wg clones fall into two classes: those that appear phenotypically normal and those that are associated with pattern duplications. Without exception (n = 53; see Experimental Procedures), clones of the first class populate only ventral portions of the legs derived from cells that normally express the *wg* gene or are positioned close to the *wg*-expressing cells (Figure 4; see Figure 3 for a diagram of the normal leg). This can be seen clearly in the tarsal segments of the second leg. As diagramed in Figure 3C, the tarsal bristles in this leg are arranged in a highly stereotyped pattern of eight columns, the ventralmost columns (4 and 5) forming characteristic thick, peg-like bristles (Hannah-Alava, 1958). Phenotypically normal clones marked bristles in columns 4 and 5 (e.g., Figure 4B) and occasionally columns 3 and 6, but never columns 1, 2, 7, or 8. These clones were found in either the anterior or posterior compartment and could extend almost the entire length of the leg (as in Figure 4), although, curiously, they never marked the most distal structure, the claw.

Conversely, clones falling into the second class, those associated with abnormal patterns, invariably contributed to lateral or dorsal portions of the leg that derive from cells that would not otherwise express *wg* (n = 183; see Experimental Procedures). As described in detail below, two features of these clones are striking. First, the *y Act5C>wg* cells contribute only a portion of the altered pattern, often just a small portion, indicating that they have the capacity to exert an organizing influence over the developmental behavior of neighboring, wild-type cells. Second, *y Act5C>wg* cells that are positioned in dorsal locations appear to form ventrolateral structures and to exert a ventralizing influence on neighboring cells.

An example of the ability of dorsally positioned *y Act5C> wg* cells to reorganize dorsoventral pattern is shown in Figure 5B. This figure shows a *y Act5C>wg* clone that marks a thin strip of dorsolateral territory extending proximodistally through the posterior compartment of the third leg. As shown in Figures 5A and 5C, the most proximal

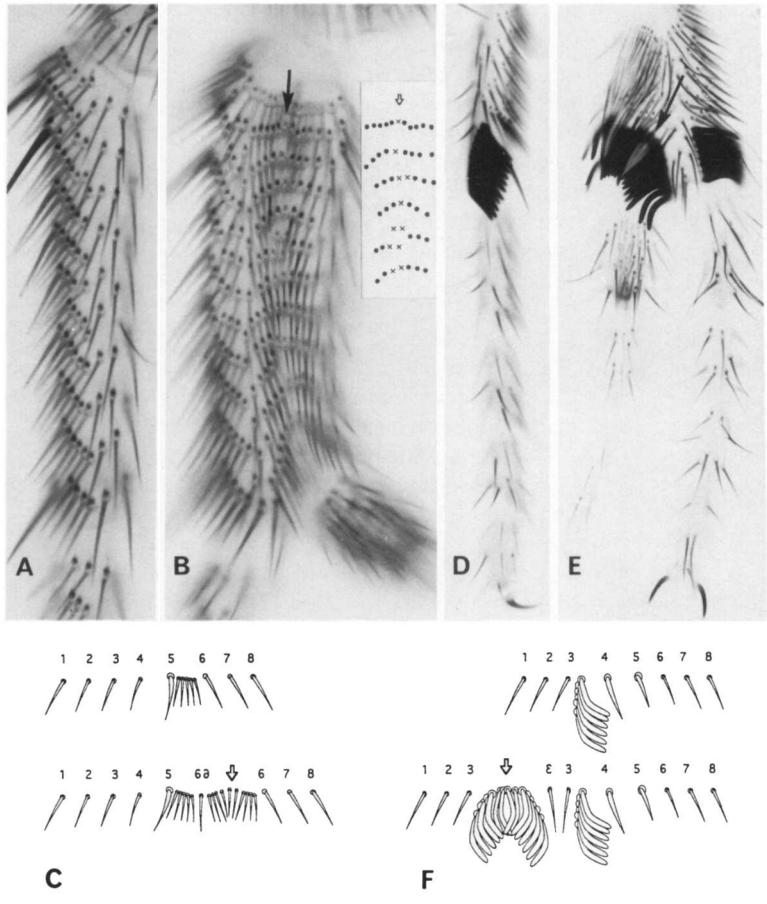

Figure 5. Dorsolateral *y Act5C>wg* Clones Organizing Ventrolateral Patterns in the First and Third Legs

(A) Posterior aspect of the basitarsus of a normal third leg showing bristle columns 5–8 as well as the transverse row bristles that lie between columns 5 and 6 (oriented as in the diagram in [C]).

(B) Dorsolaterally situated *y Act5C>wg* clone in the posterior compartment (oriented as in [A]). This clone marks a thin strip of tissue that extends proximodistally from the femur to the tarsus (arrow). The clone is associated with a mirror-symmetric pattern of adventitious transverse bristle rows (diagramed in [C]). Although the *y* marker is difficult to see in photographs of transverse row bristles that are normally only lightly pigmented, it is readily apparent on the bracts that form immediately proximal to the socket of each bristle. The insert shows a camera lucida drawing of the bracts associated with the adventitious transverse bristle rows immediately to the left (*y* bracts are indicated by x, y^+ bracts by closed circles). Marked cells belonging to the clone form transverse row bristles as well as bracts and recruit surrounding wild-type cells on each side to do the same. These adventitious transverse bristle rows do not form at the expense of the normal dorsolateral pattern but instead seem to be intercalated between normally patterned ventral and dorsolateral patterns on either side.

(C) Diagram of the normal (upper) and abnormal (lower) third leg patterns shown in (A) and (B), as well as of the position of the *y Act5C>wg* clone (arrow). The photographs in (A) and (B) show only the posterior halves of the dorsoventral pattern (bristle columns 5–8).

(D) Anterior aspect of the basitarsus of a normal male first leg showing the row of specialized transverse row bristles that first form between columns 3 and 4 but then rotate 90° to construct the sex comb (oriented as in the diagram in [F]).

(E) Dorsolaterally situated *y Act5C>wg* clone in the anterior compartment (orientated as in [D]). This clone forms a thin strip of tissue extending proximodistally from the trocanter to the tarsus. As in the clone in (B), the marked cells form ventrolateral structures (e.g., a cluster of three sex comb teeth; arrow) and have recruited wild-type cells on either side to form combs of ectopic sex comb teeth.

(F) Diagram of the normal (upper) and abnormal (lower) first leg patterns shown in (D) and (E), as well as of the position of the *y Act5C>wg* clone (arrow). The photographs in (D) and (E) show only anterior halves of the dorsoventral pattern (bristle columns 1–4).

tarsal segment (the basitarsus) of the third leg can be subdivided into dorsal, dorsolateral, ventrolateral, and ventral columns of bristles (numbered 1–8 as in the second leg in Figure 3C). However, unlike the second leg, the bristles in columns 5 and 6 of the third leg are linked by transverse rows of tightly packed bristles (Figures 5A and 5C). In the clone in Figure 5B, the *y Act5C>wg* tissue forms spatially inappropriate transverse row bristles, as do neighboring wild-type cells on either side. Thus, *y Act5C>wg* cells positioned in the dorsolateral portion of this compartment give rise to ventrolateral structures and can recruit surrounding cells to form symmetric ventrolateral patterns. A similar situation is shown in Figure 5E, in which a *y Act5C>wg* clone marks dorsolateral cells in the anterior compartment of the male first leg. As shown in Figures 5D and 5F, the ventral and ventrolateral columns of bristles in the anterior compartment of this leg (3 and 4) are linked by transverse bristle rows, one of which rotates 90° and differentiates a row of thick, heavily pigmented "sex comb teeth." In the clone in Figure 5E, *y Act5C>wg* cells positioned in the dorsolateral portion of the anterior compartment form sex comb teeth and recruit neighboring y^+ cells on either side to form sex comb teeth. Thus, as in Figure 5B, *y Act5C>wg* cells that occupy a dorsal position form ventrolateral structures and organize mirror-symmetric ventrolateral patterns in surrounding, wild-type tissue.

The dorsoventral patterning abnormalities caused by *y Act5C>wg* cells are often associated with bifurcations of the limb that generate supernumerary appendages. These bifurcations can arise at any position along the proximodistal axis of the leg (e.g., Figure 6) and in either the anterior or posterior compartment. As in the more local reorganizations of dorsoventral pattern described above, the *y Act5C>wg* cells associated with the bifurcation usually contribute to only a small portion of the altered pattern (e.g., Figures 6A and 6B). In general, these clones extend from more proximal portions of the leg, where they are sometimes associated with local alterations of dorsoventral pattern (see, for example, Figures 5B and 5E) through the point of bifurcation and into the supernumerary ap-

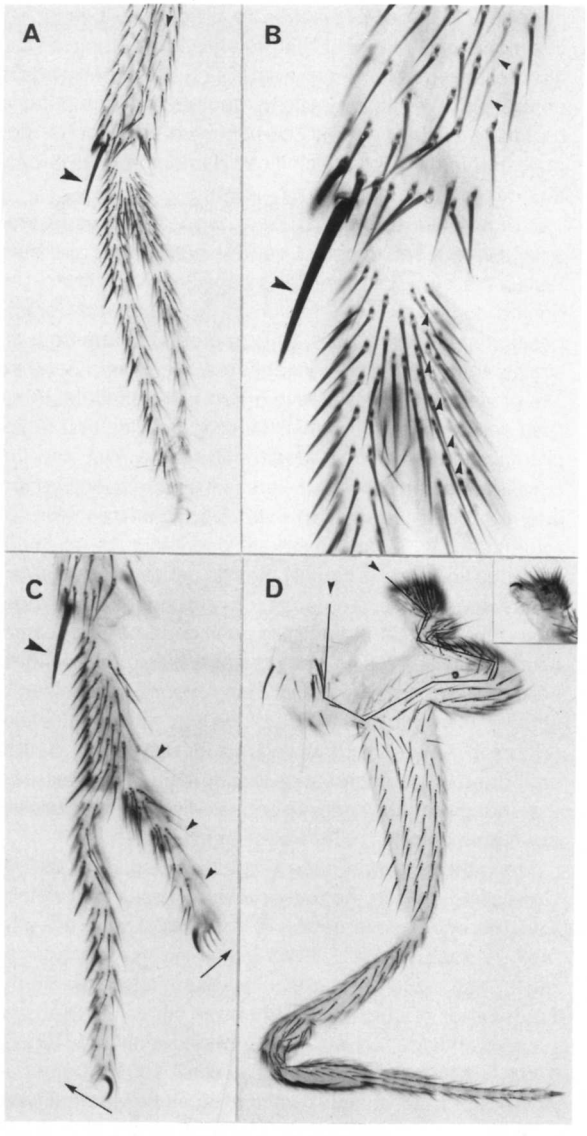

Figure 6. Supernumerary Appendages Formed in Association with *y Act5C>wg* Clones

(A and B) Posterior aspect of a second leg containing a dorsolaterally situated *y Act5C>wg* clone associated with a supernumerary limb. Large arrowhead marks the apical bristle, a ventral landmark at the distal end of the tibia. Bristle columns 5–8 in the normal basitarsus (left) are oriented as in Figure 3C. Note the characteristic thick, peg-like bristles belonging to ventral column 5. The *y Act5C>wg* clone marks only a thin proximodistal strip of tissue (small arrowhead in [A]) both proximal and distal to the point of bifurcation (shown at higher magnification in [B]). Note also that the marked tissue forms only a small portion of the supernumerary appendage (*y* bristles are marked with small arrowheads in [B]), and that the supernumerary appendage lacks the peg-like bristles typical of ventral bristle columns 4 and 5.

(C) Posterior aspect of a second leg containing a dorsally situated *y Act5C>wg* clone associated with a supernumerary appendage (right). As in the second leg shown in the same orientation in (A) and (B), thick ventral bristles are formed only in the normal limb (left; large arrowhead marks the apical bristle). The reversed dorsoventral polarity of the supernumerary appendage (right) is apparent in the orientation of the y^+ claw (arrow), which is opposite that of the y^+ claw in the normal appendage. *y* bristles belonging to the clone are marked by small arrowheads. The *y Act5C>wg* tissue also forms three rudimentary claws at the tip of the supernumerary appendage.

(D) Supernumerary appendage (right) formed proximally in association with a dorsolaterally situated *y Act5C>wg* clone. Marked bristles are present in the coxa and trocanter (see Figure 3B) proximal to the point of bifurcation in the femur as well as in the supernumerary appendage (the region of *y* tissue is outlined by a black line ending in arrowheads). The supernumerary appendage extends proximodistally from the femur to the distal tarsus, where it forms a claw (shown in inset in another plane of focus).

pendage (Figures 6A and 6B). Strikingly, the supernumerary appendages almost always lack extreme ventral structures. For example, the characteristically thick, peg-shaped bristles of the tarsal segments (columns 4 and 5) are typically absent (Figures 6A and 6C). However, the *y Act5C>wg* cells within the supernumerary appendage can form ventrolateral structures and appear to exert a ventralizing influence on surrounding cells. For example, in favorable situations, such as in the clone in Figure 5B, ventrolateral pattern elements, e.g., posterior transverse bristle rows, can be distinguished within the supernumerary limb closely associated with the clone. In addition, *y Act5C>wg* cells that arise dorsally can cause the formation of a supernumerary limb with opposite dorsoventral polarity to the normal limb (Figure 6C).

We note that supernumerary appendages typically result from grafting experiments in which dorsal and ventral portions of developing insect legs are artificially juxtaposed (Bohn, 1965, 1972a, 1972b; French, 1978, 1980). Hence, the association of dorsally situated *y Act5C>wg* cells with supernumerary limbs provides further evidence that these cells inappropriately behave like more ventrally situated cells. We also note that the supernumerary appendages associated with *y Act5C>wg* cells extend distally to varying degrees, sometimes ending within the tarsal segments (see Figure 5D) or, alternatively, extending to the distal tip, where they can form multiple claws, some *y* and some y^+ (Figure 6C). Varying degrees of distalization are also observed in supernumerary appendages obtained in other limb systems, depending on the specific rearrangements of dorsal and ventral cells (French, 1981; Bryant et al., 1981). Despite the many models devised to account for supernumerary limb formation following more classical grafting experiments (French et al., 1976; Bryant et al., 1981), it remains unclear why it is triggered by juxtaposing normally remote dorsal and ventral cells.

Ventrolateral Organizing Activity of *Act5C>wg* Cells Is Associated with Low Levels of *wg* Expression

As described above, the *wg* gene is normally expressed in cells giving rise to the most ventral derivatives of the adult legs. In contrast, dorsally situated cells expressing *wg* under the control of the *Act5C* promoter appear to form ventrolateral rather than extreme ventral structures. One possible explanation for this difference is that high levels of *wg* expression are necessary to specify ventral structures, whereas lower levels, driven by the *Act5C* promoter, specify more lateral structures. To assess the level of ectopic *wg* expression in *y Act5C>wg* clones, we subjected third instar larvae carrying both the *hsp70-flp* and *Act5C>y*$^+$*>*

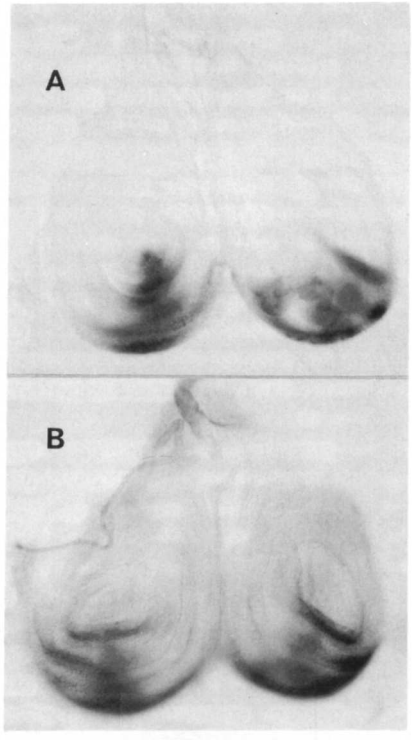

Figure 7. *wg* Expression in *Act5C>wg*-Expressing Leg Discs

Wg protein expression in first leg discs derived from control *y; hsp70–flp* (A) and experimental *y; Act5C>y⁺>wg; hsp70–flp* (B) late third instar larvae subjected to a severe heat shock (37°C for 60 min) 12 hr earlier. The left and right first leg discs are normally tightly paired during larval life. Note the ubiquitous low level *wg* expression in the experimental, but not the control, discs.

wg genes (experimentals) as well as third instar larvae carrying only the *hsp70–flp* gene (controls) to a severe heat shock (37°C for 60 min) that should have caused *flp*-mediated excision in most cells of the experimental larvae. Both the experimental and control larvae were then allowed to develop for an additional 12 hr, at which time their discs were isolated and stained for wg protein expression (see Experimental Procedures). Imaginal discs derived from both the control and experimental larvae show the normal endogenous pattern of wg protein expression. However, the experimental discs also show faint ubiquitous staining not observed in the control discs (Figure 7). Hence, it appears that the level of constitutive *wg* expression derived from the fused *Act5C>wg* gene is much lower than that of spatially regulated expression derived from the endogenous gene, supporting the view that varying levels of *wg* activity specify varying portions of dorsoventral pattern.

Discussion

Organizing Activity of Wg Protein

Embryonic organizers are distinct subpopulations of cells that are both necessary and sufficient to specify the global pattern of a larger population to which they belong. As Spemann and Mangold first established in their classic study of the amphibian dorsal lip (1924), a compelling demonstration of organizer activity requires that "the putative organizer be brought into contact with other parts, normally foreign to it, on which it can demonstrate its capacities" (as cited in Hamburger, 1964).

In Drosophila leg imaginal discs, wg protein is expressed exclusively in prospective ventral cells. Here we have tested the putative organizing capacity of wg protein by driving constitutive expression of the protein in dorsally positioned clones of cells. This approach differs from the classic transplant experiment in that we have moved the site of synthesis of a putative organizing molecule, rather than cells associated with organizing activity, to a region normally foreign to the endogenous source. We show that dorsally situated cells that express wg protein form ventrolateral structures and exert a ventralizing influence on surrounding cells. Thus, the ectopic expression of *wg* in dorsally positioned cells causes them to behave as secondary ventral organizers. In contrast, driving constitutive expression of wg protein in ventrally positioned cell clones has no effect on the final pattern, as would be expected if these cells already constitute the native ventral organizer by serving as a source of endogenous wg protein expression (Figure 8). A ventral organizing role for wg protein is further supported by the observation that mutations that decrease the endogenous *wg* activity cause deletions of ventral leg structures (Baker, 1988; Peifer et al., 1991).

The parallel between the amphibian dorsal lip and *wg*-expressing cells in the imaginal leg discs is also notable because ectopic expression in Xenopus of several *wg* homologs, such as *Wnt-1*, *XWnt-8*, and *wg* itself, can trigger the formation of secondary body axes in otherwise normal embryos or restore axial pattern in ultraviolet-irradiated embryos (McMahon and Moon, 1989; Smith and Harland, 1991; Sokol et al., 1991; Christian et al., 1991; Chakrabarti et al., 1992). However, it remains uncertain whether a Wnt protein is responsible for the classical organizer activity in this system. First, in the experiments of Spemann and Mangold (1924), organizer activity was associated with the presence of graft cells in the notochord and floor plate of the secondary axis. However, none of the cells in the secondary axis caused by microinjection of *Wnt-1* and *XWnt-8* transcripts into single vegetal blastomeres actually derive from the injected cell (Smith and Harland, 1991). Instead, the *Wnt-1* and *XWnt-8* activities appear to act within prospective endodermal cells to create an ectopic Nieuwkoop center that in turn triggers organizing activity in neighboring mesodermal tissue. Second, *XWnt-8* is not normally expressed in the dorsal lip (Christian et al., 1991; Smith and Harland, 1991; Sokol et al., 1991), nor has a dorsal lip–specific *Wnt* homolog been identified (reviewed by McMahon, 1992). By contrast, our results argue that ventral cells in the Drosophila leg discs organize the pattern of neighboring lateral and dorsal cells because they provide a local source of wg protein: the endogenous organizing signal. It is intriguing that the classical organizer activity defined by Spemann and Mangold was frequently

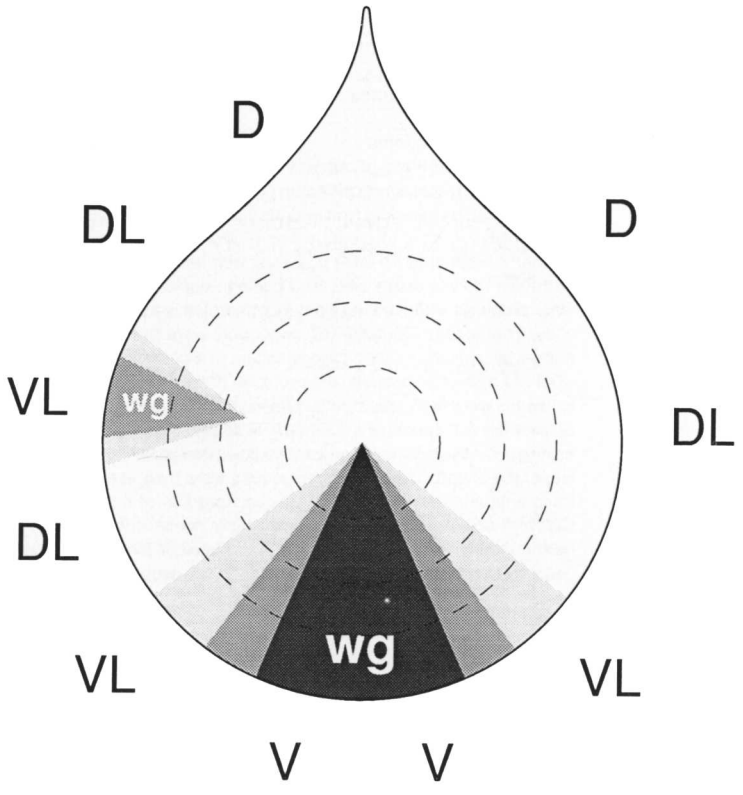

Figure 8. Organization of Dorsoventral Leg Pattern by *wg*-Expressing Cells

A model for the control of dorsoventral pattern by wg protein in the leg disc. In wild-type discs, ventrally positioned cells serve as a localized source of wg protein (dark stippling) that diffuses to or is transported to neighboring cells (lighter stippling), possibly forming a gradient. High levels of wg protein direct the formation of extreme ventral structures, lower levels direct the formation of ventrolateral structures, and more dorsal structures arise in the absence of wg protein. Accordingly, ectopic expression of low levels of wg protein in dorsolaterally situated cells would cause them to develop into spatially inappropiate ventrolateral structures, and movement of the protein to neighboring cells would account for the ability of the ectopic *wg*-expressing cells to exert a ventralizing influence on the surrounding wild-type tissue.

associated with the presence of donor cells in the notochord and floor plate. More recent experiments (Yamada et al., 1991) suggest that these tissues exert a potent ventralizing influence on neural differentiation within the spinal cord. Hence, the ventral organizing activity of *wg*-expressing cells in the leg disc may be analogous to that of the notochord and ventral floor plate cells in the vertebrate spinal cord.

The Role of *wg* in Organizing Dorsoventral Limb Pattern

Two classes of mechanisms are usually invoked to explain how small populations of cells exert an organizing influence over the pattern of a much larger field. In the first class, these regions serve as the source of a morphogen that can diffuse or be transported through a tissue, generating a gradient that triggers distinct cellular behaviors in response to different threshold concentrations (e.g., Dalcq, 1938; Lawrence, 1972; Sander, 1975; Tickle et al., 1975). In the second class, these regions provide local signals that initiate a sequence of inductive events each one of which depends on single threshold responses to qualitatively distinct signaling molecules (e.g., Spemann, 1938; Rose, 1952; Jacobson and Sater, 1988; Tomlinson, 1988). Given the apparent local nature of *wg* signaling in Drosophila embryos (Dinardo et al., 1988; Martinez Arias et al., 1988; Immerglück et al., 1990; van den Heuvel, 1989; González et al., 1991; reviewed by Nusse and Varmus, 1992) as well as the possible existence of other local signals operating in this system (e.g., Ingham et al., 1991), such sequential inductive mechanisms have generally been favored as explanation of the role of *wg* in generating cell pattern (e.g., Martinez Arias, 1989; Ingham et al., 1991; but see Bejsovec et al., 1991; Bejsovec and Peifer, 1992). However, as we describe below, our data on the ventral organizing activity of ectopic *Act5C>wg* cells support the possibility that a gradient mechanism may be involved.

The key result that can be interpreted in terms of a gradient mechanism is our finding that the level of *Act5C>wg* expression that directs ventrolateral patterning in dorsal portions of the leg appears to be low relative to the level of endogenous gene expression in ventrally positioned cells (Figure 7). Hence, one could argue that high levels of *wg* activity specify ventral pattern, low levels specify lateral pattern, and no expression leads by default to dorsal pattern (Figure 8). Consistent with this view, Peifer et al. (1991) have found that mutations that reduce endogenous *wg* function appear to cause ventral portions of the leg pattern to be dorsalized. Because the *wg* gene is expressed at high levels in a discrete subset of ventral leg cells and the protein is likely to be secreted, these cells could serve as a local source for a concentration gradient of wg protein.

The organizing activity of *wg*-expressing cells in the Drosophila leg appears analogous to that of the zone of polarizing activity (ZPA) in the chick limb bud. In this case, Tickle (1981) has shown that when ZPA cells derived from the posterior margin of a donor bud are transplanted to the

anterior margin of a recipient bud, the resulting limb has a mirror-symmetric 432234 digit pattern (reading from anterior to posterior, where the normal pattern is 234). However, the pattern and extent of digit duplication depends on the number of transplanted ZPA cells: when the number is reduced in 2- to 3-fold increments, the resulting limbs show 32234, then 2234, and finally the normal 234 digit patterns. These results have been interpreted in terms of a gradient mechanism (Tickle et al., 1975) in which the ZPA cells constitute the source of a diffusible morphogen that specifies different digits as a function of concentration. Further, they argue against cascade-type models of sequential induction because they indicate that low levels of the ZPA signal can specify either digits 2 or 32 without formation of digit 4. The same reasoning can be applied to the organizing activity of wg protein in the leg imaginal discs. The ability of low levels of ectopic *Act5C>wg* expression to organize ventrolateral pattern without specifying more extreme ventral pattern elements is equivalent to the ability of low levels of the ZPA signal to organize digit patterns 2 or 32 without specifying digit 4. As in the chick limb bud, this result can be taken as an argument against a sequential induction mechanism.

The possibility that *wg* functions as a gradient morphogen raises the question of its range and signaling potential. In particular, the sector of detectable *wg* expression in mature imaginal leg discs comprises only around 10%–20% of the disc. Hence, assuming that a similar relationship holds when the disc first forms, the realm of direct *wg* action might be only a relatively small portion of the disc. It may be useful to compare this situation with what is known about the *bicoid* (*bcd*) morphogen gradient in early embryos. The realm of direct *bcd* action appears to be restricted to the anterior half of the body (Dreiver and Nüsslein-Volhard, 1988a, 1988b; Struhl et al., 1989; Driever et al., 1989). However, under certain conditions, the *bcd* gradient can organize virtually the entire body pattern (Hülskamp et al., 1990). In this case, control over the posterior half of the body is exerted indirectly, through the ability of *bcd* to initiate a cascade of gradients of other morphogens, such as hunchback, Krüppel, knirps, and giant proteins, which operate within the posterior half of the body (Struhl et al., 1992). Similarly, graded *wg* activity in the ventral portion of the leg disc might influence lateral and dorsal patterning indirectly, by regulating the formation of additional morphogen gradients that operate in more dorsal portions of the disc.

If *wg* exerts a global influence on pattern by indirectly regulating the activities of other signaling molecules that function at a distance, how can we distinguish between gradient and sequential-induction models of *wg* function? As in the case of the *bcd* morphogen, we argue that the key distinction is that gradient models demand that quantitative differences in the concentration of wg protein, a single molecular species, trigger different outcomes, whereas in sequential-induction models, different outcomes require the serial activation of qualitatively distinct signaling molecules. Hence, a clear prediction of the gradient hypothesis is that higher levels of constitutive *wg* expression should lead to the specification of ventral rather than ventrolateral structures at ectopic positions.

Experimental Procedures

Plasmid Constructions

The following general strategy was used for constructing each hybrid gene. A *P*(*ry*$^+$) transformation vector, *C20* (Rubin and Spradling, 1983), was modified by inserting NotI and XbaI linkers in the unique SalI and HpaI polylinker sites to create plasmid *C20NX*. As described below, promoter fragments (*P*) were prepared with flanking NotI (upstream) and KpnI (downstream) sites and coding sequence fragments (*CS*) were prepared with flanking KpnI (upstream) and XbaI (downstream) sites. The desired *C20NX[P-CS]* constructs were then generated by three-way ligation, in each case resulting in a plasmid with a unique KpnI site at the join between the promoter and structural gene. *flp*-out cassettes were then prepared by placing the sequences to be deleted between direct repeats of a minimal FRT sequence, the upstream FRT having a KpnI site at the 5′ end and the downstream FRT having a KpnI site at the 3′ end. The resulting cassettes were then excised as KpnI fragments and inserted into the unique KpnI site of the appropriate *C20NX[P-CS]* plasmids prepared previously. As described below, we had to modify this general strategy in the case of the *Act5C>y*$^+$*>wg* gene by substituting NheI sites for KpnI sites because the *y*$^+$ gene contains two internal KpnI sites.

Promoters

hsp70

NotI and KpnI linkers were inserted at the 5′ and 3′ ends of a standard *hsp70* promoter fragment (Lis et al., 1983; Struhl, 1985).

Act5C

A NotI linker was inserted at the EcoRI site positioned ~4.4 kb upstream of the translational start of the *Act5C* gene (Bond and Davidson, 1986). A set of oligonucleotides kindly provided by B. Bond was then used to insert a KpnI site in place of the initiator ATG (*Act5C* = CTTACAAAATG; modified *Act5C* = CTTACAGGATCGGGTACC). The resulting NotI–KpnI fragment constitutes the *Act5C* promoter fragment shown in Figure 1.

Coding Sequences

flp

Appropriate oligonucleotides were used to replace the 5′ noncoding portion and initiator ATG of the *flp* gene (Harley and Donelson, 1980) by the sequence GGTACCCAAAAAAATG that creates a KpnI site as well as a poly(A) stretch just upstream of the initiator ATG. The 3′ noncoding portion of the *flp* gene was then fused at the FspI site 102 bp downstream of the stop codon to a 2.1 kb SalI–EcoRI fragment containing the 3′ untranslated region (UTR) of the *hsp70* gene (Udvardy et al., 1985).

nuc-lacZ

A KpnI site was created at the XbaI site immediately upstream of the *nuc-lacZ* gene constructed by G. Riddihough (*pβn27.1*, Riddihough and Ish-Horowicz, 1991) by blunting this site as well as an upstream Asp718 site with Klenow polymerase and then fusing the two ends with ligase. An XbaI linker was then inserted at the SalI site immediately downstream of the *hsp27* 3′ UTR positioned just downstream of the stop codon in this hybrid gene.

wg

A 1.8 kb FnuII–AccI fragment derived from the *wg* cDNA clone SP6-wg (Rijsewijk et al., 1987) that includes the entire coding sequence flanked by 140 bp of 5′ noncoding sequence and 80 bp of 3′ noncoding sequence, was inserted into the BamHI and HincII sites of a polylinker derived from the puc19 and pSP64 plasmids such that KpnI and SmaI sites are positioned immediately upstream of the 5′ FnuII site and PstI and HindIII sites are positioned immediately downstream of the 3′ AccI site. A DNA fragment containing the 3′ UTR and transcriptional terminating sequences from the *α1 tubulin* gene (Theurkauf et al., 1986; Lawrence et al., 1987) was then fused downstream of the HindIII site at the 3′ end of the *wg* coding sequence. The 3′ end of the *α1 tubulin* DNA contains an artificial XbaI site allowing the *wg* coding sequence and *α1 tubulin* trailer to be excised as a KpnI–XbaI fragment.

flp-Out Cassettes

>Draf$^+$>

A minimal cis-acting FRT seqence was obtained from the plasmid J3 (Jayaram, 1985) and used to generate the plasmid J32 in which a NheI–BamHI stuffer fragment is positioned between direct repeats of the minimal FRT, which in turn are flanked by KpnI sites. (The resulting sequence is GGTACCCGGGGATCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGCGCTTTTGAAGCTAGC . . . GGATCCGGGGGATCTTGAAGTTCCTATTCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCAGAGCGCTTTTGAAGCTGGGTACC; the KpnI, NheI, and BamHI sites are underlined.) In a separate construction, a 2.1 kb SalI–BamHI fragment containing the 3′ UTR and transcriptional terminating sequences from the *hsp70* gene (Udvardy et al., 1985) was inserted into the polylinker of puc19, and the HindIII site of the polylinker was converted into an NheI site by cutting, blunting, and religating. The resulting NheI–BamHI fragment containing the *hsp70* 3′ UTR was then inserted into the unique NheI and BamHI sites of plasmid J32 to create plasmid J33. As diagramed in Figure 1, the *hsp70* trailer sequences fall between direct repeats of the minimal FRT, which are flanked in turn by KpnI sites. A 4.4 kb BamHI genomic fragment containing the entire *Draf*$^+$ gene (Nishida et al., 1988) was inserted into the unique BglII site within the *hsp70* 3′ trailer of J33, the resulting KpnI fragment (see Figure 1) constituting the >*Draf*$^+$> cassette.

>y$^+$>

The *y*$^+$ gene is contained within a 7.7 kb SalI–BglII genomic fragment that contains two KpnI sites, both of which cut within noncoding sequences, as well as an internal BglII site that cuts within the coding sequence (Geyer and Corces, 1987). This DNA was initially inserted into the polylinker of a plasmid vector so that an artificial SalI site is positioned just downstream of the BglII site at the 3′ end of the *y*$^+$ gene. Because there are no internal SalI sites in the SalI–BglII genomic fragment, this allowed us to excise the *y*$^+$ gene as a 7.7 kb SalI–SalI fragment. We then modified the J33 plasmid by first inserting a NotI linker between the unique NheI and SalI sites (thereby destroying both sites), then changing the unique EcoRI site downstream of the *hsp70* 3′ UTR to a SalI site, and finally creating NheI sites on each side of the KpnI–KpnI fragment that contains the >> *flp*-out cassette. The plasmid J35, which contains the >*y*$^+$> *flp*-out cassette shown in Figure 1, was then created by inserting the SalI–SalI fragment containing the *y*$^+$ gene into the modified J33 plasmid. To insert this *flp*-out cassette into the *C20NX, Act5C–wg* gene construct, we replaced the unique KpnI site in this construct with a unique NheI site and then inserted the NheI–NheI >*y*$^+$> cassette from J35. As noted in Figure 1, we were unable to generate *Act5C>y+>wg* transformants when the >*y*$^+$> cassette was oriented with the *hsp70* transcriptional terminating sequences immediately downstream of the promoter proximal FRT. Transformants were obtained, however, when the cassette was inserted in the opposite orientation (see Figure 1). In this case, transcription from the *Act5C* promoter is presumably terminated at a cryptic site within the *y*$^+$ or *hsp70* DNA.

Generation of *Act5C>nuc-lacZ* and *Act5C>wg* Clones in the Imaginal Discs

The *hsp70–flp*, *Act5C>Draf*$^+$*>nuc-lacZ*, and *Act5C>y*$^+$*>wg* genes were inserted into the genome by P element–mediated transformation. Stocks homozygous for each of the transduced genes were then generated by standard genetic crosses.

Flies homozygous for the *hsp70–flp* gene were crossed to flies homozygous for either the *Act5C>Draf*$^+$*>nuc-lacZ* or *Act5C>y*$^+$*>wg* gene, and staged progeny were subjected to heat shock at varying times during development, as described above (heat shocks were applied by partially immersing agar plates or tubes containing larvae in a water bath at the appropriate temperature). The homozygous *hsp70–flp* and *Act5C>y*$^+$*>wg* stocks were also mutant for the endogenous *y* gene to allow loss of the *y*$^+$ marker gene to be detected following *flp*-mediated recombination. As described in Results, the efficiency of clone induction depends on the severity of the heat shock applied, which itself depends on the temperature and duration of the shock.

Because *Act5C>wg* clones can cause abnormal patterns in the imaginal disc derivatives, which in turn can prevent eclosion of the adult fly, there is a potential bias in our experimental sample. To assess this bias, we used the compound microscope to screen for *y* clones on the legs of 43 *Act5C>y*$^+$*>wg/hsp70–flp* flies derived from larvae that had been heat shocked for 20 min at 32°C during the first larval instar (aged 24 ± 2 hr after fertilization). A total of 69 clones were found, of which 40 were in anterior and 29 were in posterior compartments. Of the 69 clones, 53 appeared phenotypically normal and were restricted to ventral portions of the leg (see Figures 3 and 4), whereas the remaining 16 were associated with pattern duplications and populated lateral or dorsal portions of the leg.

In the main experiment, larvae aged for varying times between 1 and 3 days after fertilization (i.e., during the first and second larval instars) were subjected to a single mild heat shock (either 30°C or 32°C for 20–40 min). Surviving flies were then screened for abnormal legs under the dissecting microscope, and the legs were dissected and mounted in a 1:1 mixture of Canada balsam and methyl salicylate for examination under the compound microscope. In 167 of 175 legs, the abnormal pattern was associated with *y* tissue that populated lateral or dorsal regions (in the remaining 8 cases, only a small region of the leg pattern was abnormal, and no *y* bristles were observed). Of the 167 clones, 66 were anterior compartment clones, 75 were posterior compartment clones, and 26 could not be unambiguously classified as anterior or posterior owing to the disruption of the normal pattern. Taken together with the 16 cases of *y Act>wg* clones associated with abnormal patterns described in the preceding paragraph, a total of 183 *y Act>wg* clones associated with abnormal patterns were examined.

The association of *y* ectopic *wg*-expressing cells with abnormal limb pattern is specific to the expression of the *wg* coding sequence and not a consequence of the *flp* recombination method. We performed similar experiments using the coding sequence for the *scute* (*sc*) gene instead of *wg*. *y Act5C>sc* clones are readily generated and found throughout the imaginal disc derivatives, but are not associated with alterations in limb pattern. Instead, they are often associated with additional bristles (always formed by *y Act5C>sc* cells within the clone). A similar phenotype is also observed when ectopic *sc* expression is generated by mutations that alter the normal regulation of *sc*, or when the *sc* coding sequence is expressed ubiquitously under the control of the *hsp70* promoter (Balcells et al., 1988; Romani et al., 1989; Rodríguez et al., 1990).

Immunohistochemistry and Immunofluorescence

Mature third instar larvae were cut in half in BSS buffer (Chan and Gehring, 1971) and the anterior half-carcasses to which the thoracic and eye-antennal discs are attached were turned inside out. The carcasses and attached discs were then fixed in 4% formaldehyde in PEM buffer (0.1 M PIPES [pH 7.0], 1 mM $MgSO_4$) for 20 min, rinsed in BSS, and then transferred to PBT (1% bovine serum albumin, 0.1% Triton X-100, in phosphate-buffered saline). Immunohistochemistry and immunofluorescence, beginning with incubation in primary antisera, were performed using standard protocols for embryos. Primary antisera were rabbit α β-galactosidase (Cappel) and α wg (van den Heuvel et al., 1989) polyclonal antibodies, and mouse α β-galactosidase (Promega) and α en (Patel et al., 1989) monoclonal antibodies. Secondary antisera were appropriate horseradish peroxidase–, Texas red–, and fluorescein–conjugated antibodies. Following the staining reaction, the carcasses and attached discs were washed in phosphate-buffered saline plus 0.1% Triton X-100 and transferred to 80% glycerol, after which the discs were recovered by dissection and mounted in 80% glycerol for compound or confocal microscopy.

Act5C>wg expression was assayed in the imaginal discs as follows. Control *y; hsp70–flp*, and experimental *y; Act5C>y*$^+$*>wg; hsp70–flp* third instar larvae were heat shocked in parallel (60 min at 37°C), allowed to develop an additional 12 hr, and then dissected as described above, except that the control larvae were initialiy cut into disproportionally large anterior and small posterior halves before the anterior halves, to which the discs attach, were turned inside out. Control and experimental discs were then processed together in the same tube for immunohistochemical staining and were distinguished at the end of the procedure by the size of the larval carcass to which each was attached.

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